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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Abnormal chromosome number is a phenotype characteristic for most of the cancer cells. Thus, it may be a direct cause of human cancer including breast cancer. In this research project, we aim to test this hypothesis by abrogating the spindle checkpoint that is a major surveillance mechanism responsible for maintenance of the normal chromosome number. p55CDC serves as a target of the checkpoint. If it is unable to bind to a spindle checkpoint protein, Mad2, it abrogates the checkpoint in a dominant manner. We have generated several p55CDC mutants that have lost the ability to interact with Mad2, but otherwise normal. These mutants, when expressed in Hela cells, abrogate the spindle checkpoint expectedly. In BJ cell line, they are however toxic and we failed to establish an experimental system to test if a loss of the checkpoint causes chromosome instability and neoplasmic transformation. As an alternative strategy, we propose overexpression of Cmt2. Cmt2 is a novel Mad2-binding protein we have identified recently. Our characterization indicates that Cmt2 may directly interact with the p55CDC-Mad2 complex and promote dissociation of the complex. In the normal cell cycle, it may play a role as a silencer of the checkpoint.

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Introduction: Abnormal karyotype is a hallmark of cancer cells, including breast cancer. Therefore, it has been postulated that chromosome instability may be a direct cause of the genesis/stage progression of cancer. The spindle checkpoint, that delays the onset of sister chromatid separation until all the kinetochores capture the mitotic spindle, plays an important role for maintenance of the normal chromosome number. We suspect that loss of the checkpoint may produce aneuploids which serve as the epicenter of cancer. To test this hypothesis, we propose to abolish the checkpoint and examine the phenotypes.

The heart of the mechanism of the checkpoint is that Mad2, a component of the checkpoint, binds to its target, human p55CDC, and prevents it from promoting proteolysis prerequisite to sister chromatid separation. If Mad2 is unable to bind to p55CDC, the checkpoint is not functional. On the basis of this principle, we have proposed to mutate the Mad2-binding domain of p55CDC and generate mutants of p55CDC defective in binding to Mad2. If properly mutated, expression of such mutants would abrogate the checkpoint in a dominant manner. In year 1, we have generated a number of mutants of p55CDC to which Mad2 may not bind in the yeast two-hybrid system. In the Year 2, we have further characterized these mutants *in vivo* and found that some of these p55CDC mutants indeed cannot bind to Mad2 and abolish the function of the spindle checkpoint in Hela cells.

In the Year 3 (July 2001 – June 2002), we have continued the study to characterize these mutants *in vivo*. In addition, a novel Mad2-binding protein, Cmt2, has been the subject for the molecular study.

Body:

1. Expression of p55CDC mutants in BJ cell lines: In the Year 2, we showed that three mutants of p55CDC, M4, M9 and M45, abrogate the spindle checkpoint in Hela cells. In order to test if a loss of the checkpoint can cause chromosome instability and neoplasmic transformation, these mutants have been introduced into BJ cells. This cell line is considered to be normal and cannot grow in an anchorage-independent manner on the soft agar media. It has been shown that the cell line becomes competent to grow on the soft agar media upon neoplasmic transformation.

Each p55CDC mutant was placed under the control of a tet-inducible system and transformed into BJ cell line. Although the same construct was efficiently transformed into the Hela cells, it was not so when BJ cell line was used as the host. Because the vector which did not carry the p55CDC mutant yielded the transformamts normally, the low

efficiency of the transformation is due to the presence of the p55CDC mutant, which is probably expressed at a low level even when the tet-inducible system is turned off.

Five transformants by the construct containing the p55CDC mutant (M45) were analyzed for 1) expression of the p55CDC mutant and 2) function of the spindle checkpoint. The results indicated that three of them did not express the mutant p55CDC when the tet-inducible system was turned on. The other two transformants expressed the mutant allele. They, however, maintained the functional spindle checkpoint. These results strongly suggest that a very low level of the expression of the mutant allele is toxic to BJ cell line. The five transformants we obtained either carry a secondary mutation which suppresses the mutant allele or have lost the ability to express the mutant allele.

2. Characterization of a novel Mad2-binding protein, Cmt2: In the Year 2, we identified a novel Mad2-binding protein, Cmt2, through a screen by the yeast two-hybrid assay. As shown below, characterization of Cmt2 suggested that Cmt2 may interact with Mad2 for silencing the spindle checkpoint.

2-1. Cmt2-Mad2 complex in mid-mitosis: First, we examined at which stage of the cell cycle Cmt2 forms a complex with Mad2. Culture of Hela cells was synchronized by double thymidine block & release and cell extracts were prepared for immuno-precipitation with the antibody to Mad2. At early mitosis, the majority of Mad2 appeared to form a complex with p55CDC (Figure 1, IP: α-MAD2 probed with α-55CDC, lanes 8-10). A minor fraction of Cmt2 was also found to from a complex with Mad2 at these time points. As the cell cycle progressed to mid-mitosis, Mad2 no longer formed the complex with p55CDC. Coincidentally, the great majority of Mad2 bound to Cmt2 (Figure 1, IP: α-MAD2 probed with α-CMT2, lanes 10-12). It should be noted that while the level of p55CDC measured by western blot (Figure 1, top panel) reached a peak around lanes 10-12, the level of p55CDC associated with Mad2 reached a peak earlier (Figure 1, IP: α-MAD2 probed with α-55CDC, lanes 8-10). The result indicates that p55CDC that is not in the complex with Mad2 exists around mid-mitosis and that this form of p55CDC probably represents an active form responsible for promoting APC-dependent ubiquitination. The level of p55CDC measured by western blot decreased in late mitosis (lanes 13-15) due to probable destruction at or around anaphase. The result indicates that there are at least two different protein complexes containing Mad2. The one, Mad2-p55CDC, is formed and disassembled first. The formation of the Mad2-Cmt2 complex follows and becomes prominent in mid-mitosis.

2-2. Overexpression of Cmt2: The above result suggested that Mad2 switches its binding partner from p55CDC to Cmt2 in mid-mitosis. We suspected that Cmt2 may interact with the p55CDC-Mad2 complex and disassemble it when spindle formation is completed. Perhaps, Cmt2 plays a role as a silencer of the spindle checkpoint in the normal mitosis. To test this possibility, Cmt2 was overexpressed in Hela cells that were arrested by the checkpoint. The CMT2 gene was placed under the control of the tet-inducible system. Upon addition of Dox (doxycyclin, analog of tetracyclin), the system allows over-expression of Cmt2. Transfected Hela cells were blocked by thymidine and released into media that contained nocodazole.

When Dox was not added (thus Cmt2 was not overexpressed), the mitotic index, (percentage of the nonadherent pseudo-mitotic cells in population) was approximately 60 %, 14 hours after release from the thymidine block (Figure 2B). Continued incubation with nocodazole resulted in an increase of the mitotic index up to 80 %. Examination of the levels of human securin and p55CDC, both of which normally drop in mid-mitosis (Zou, et al., 1999; Weinstein, 1997), indicated that they remained stable under this condition, indicating the cells were tightly arrested due to the effect of nocodazole (Figure 2C).

When Dox was added 14 hours after release from the thymidine block, the induced Cmt2 became detectable within 3-4 hours (Figure 2D). The level of the induced Cmt2 was approximately 10-fold of the authentic Cmt2. Upon the addition of Dox, the mitotic index dropped (Figure 5B). The levels of p55CDC and securin also dropped around 8 hours after the addition of Dox (Figure 2D). These results indicate that induction of Cmt2 abrogates the function of the spindle checkpoint and overcomes the cell cycle arrest caused by the spindle poison. The overexpression of Cmt2 most likely affects the spindle checkpoint in a direct manner. Immuno-precipitation by the antibody to Mad2 demonstrated that the p55CDC-Mad2 complex was disassembled after the induction of Cmt2 while it persisted if Cmt2 was not induced (Figure 2E). We also measured DNA content of individual cells. If Cmt2 was not induced, most of the cells contained 4N content 24 hours after release from the thymidine block, indicating that they were arrested in G2 or mitosis (Figure 2F, 24 hours, -Dox). Continued presence of nocodazole resulted in appearance of cells with less than 2N content (48 hours, -Dox), probably reflecting cell death due to prolonged arrest by nocodazole. In contrast, the overexpression of Cmt2 resulted in appearance of cells with 8N content (Figure 2F, 24 and

48 hours, +Dox), indicating that the next round of DNA synthesis was completed without the previous chromosome segregation. This phenotype is typical in cells lacking the functional spindle checkpoint. It should be noted that overexpression of Cmt2 prevents appearance of cells with less than 2N content, suggesting rescue of cells from cell death due to prolonged arrest by nocodazole. We examined 11 independent cell lines in which the inducible CMT2 construct was integrated and found that they all consistently exhibited this phenotype.

Cytological observation of the cells indicated that overexpression of Cmt2 in cells arrested by nocodazole caused extensive abnormality in chromosome morphology. The great majority of cells (~ 80 %) exhibited nuclei which were larger than those of normal mitotic cells (Figure 2G left panel). It was also noticeable that they occasionally contained multiple nuclei (Figure 2G, middle panel). In addition, a minor fraction of the cells (20 %) showed partially and unequally segregating nuclear mass (Figures 2G, right panels).

Key Research Accomplishment:

In the annual report 2001, we proposed the following two changes;

1) The original tasks 5 – 14 were to characterize the mutant p55CDC in the mouse model system. By using BJ cell line, we can avoid the animal experiments to test if the mutant p55CDC causes chromosome instability and neoplasmic transformation. In the Year 3, we have followed the modified proposal and found that BJ cell line is much sensitive to the mutant p55CDC.

2) We proposed to overexpress Cmt2 to inactivate the spindle checkpoint. In the Year 3, we further characterized the Cmt2-Mad2 comple and the phenotypes associated with overexpression of Cmt2.

Reportable Outcomes:

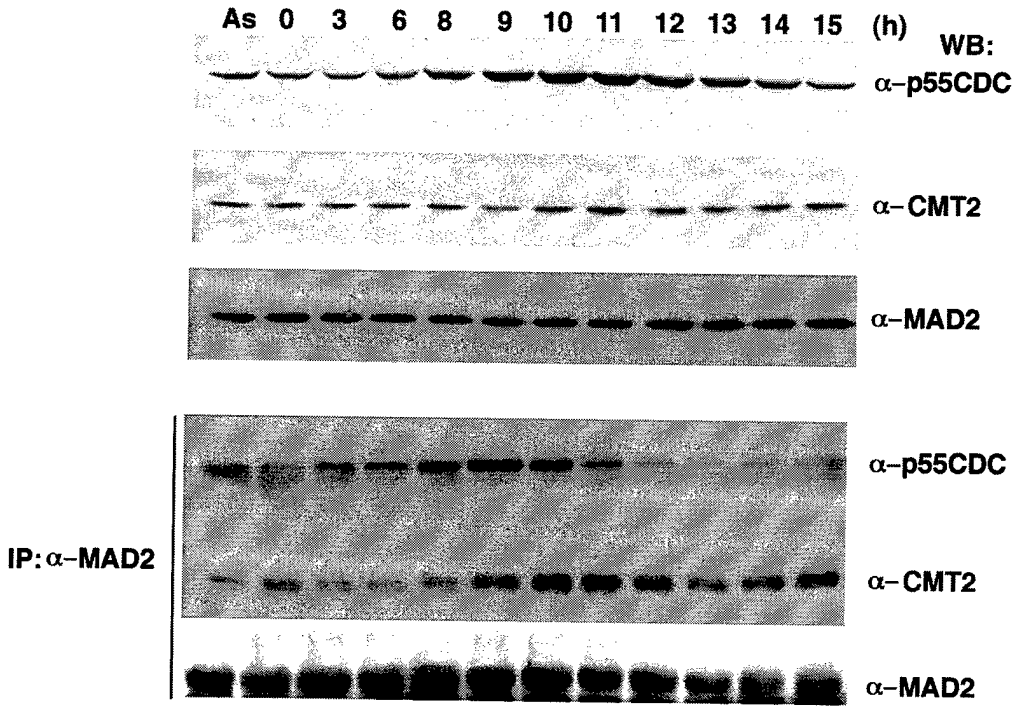
Toshiyuki Habu, Sang Hoon Kim, Jasminder Weinstein and Tomohiro Matsumoto. Identification of a Mad2-Binding Protein, Cmt2, and its Role in Mitosis. (submitted in July 2002)

Conclusion: Although we found that BJ cell line is much sensitive to the mutant p55CDC and the current strategy cannot be used to inactivate the spindle checkpoint in BJ cell line. We should consider another inducible system which is more tightly regulated or another cell line which is not too sensitive. The most prominent product in the Year 3 is

characterization of mt2, a novel Mad2-binding protein. Because it abroogates the checkpoint when it is overexpressed at a level 10 to 20-fold higher than the native locus, it can be used as an alternative of the mutant p55CDC.

Appendix: Figure 1 and 2 are attached in the following pages.

Figure 1


As 0 3 6 8 9 10 11 12 13 14 15 (h)
WB:
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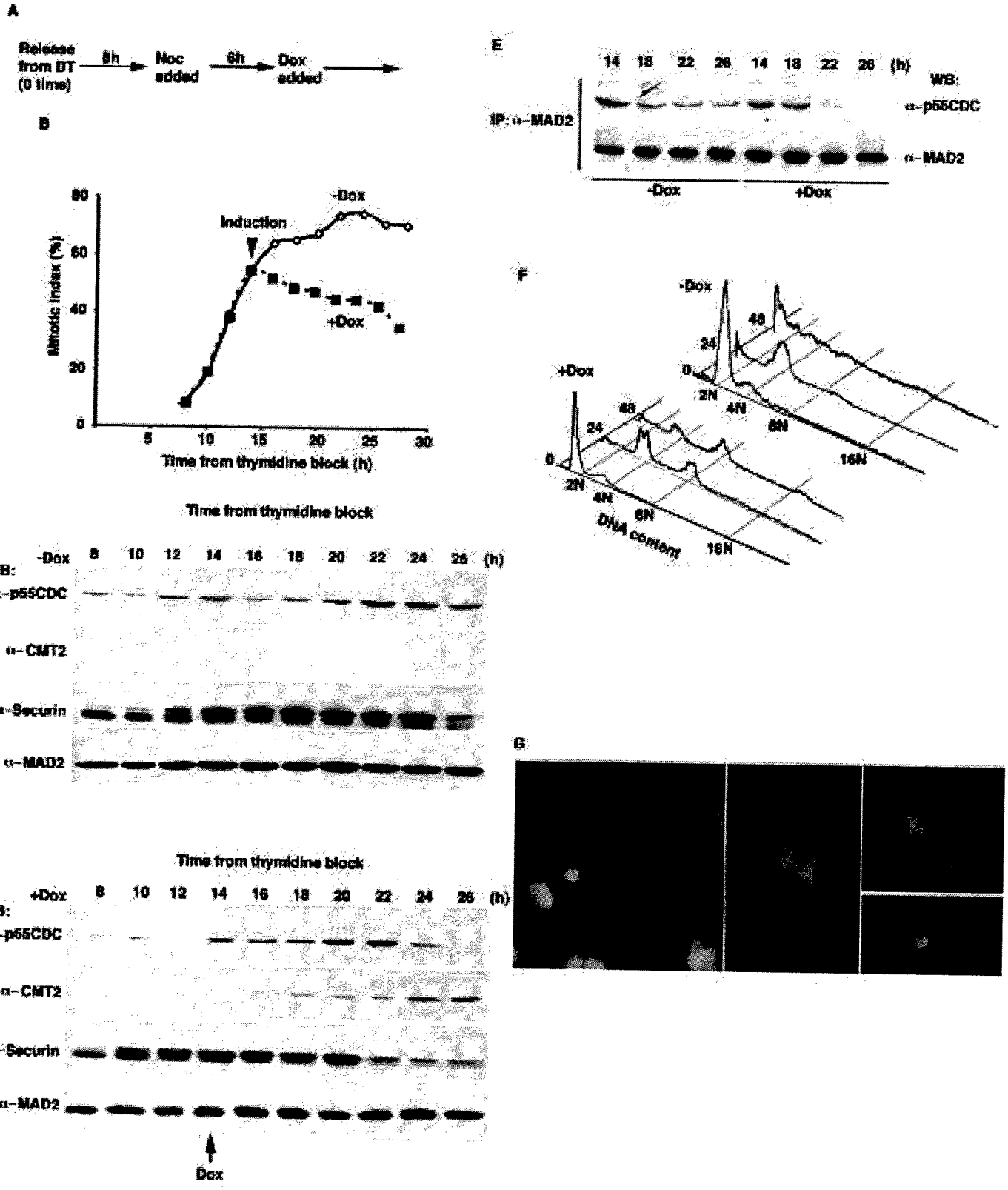
α-CMT2
α-MAD2
IP: α-MAD2
α-p55CDC
α-CMT2
α-MAD2

Figure 2


A
Release from DT (0 time)
8h
Noc added
8h
Dox added
B
Mitotic index (%)
Induction
-Dox
+Dox
Time from thymidine block (h)
C
Time from thymidine block
-Dox
WB:
α-p55CDC
α-CMT2
α-Securin
α-MAD2
D
Time from thymidine block
+Dox
WB:
α-p55CDC
α-CMT2
α-Securin
α-MAD2
Dox
E
IP: α-MAD2
WB:
α-p55CDC
α-MAD2
-Dox
+Dox
F
-Dox
+Dox
2N
4N
8N
16N
DNA content
G

Legends for Figures

Figure 1: Cell cycle analysis. Cell extracts were prepared at indicated time points after the release from double thymidine block and processed for western blot (upper 3 panels) or immunoprecipitation with the antibody to Mad2 followed by western blot (lower three panels). Extracts prepared from an asynchronous culture (AS) were also examined in the same way.

Figure 2. Overexpression of Cmt2

(A) Hela cells in which Cmt2 was inducible by Dox were arrested by double thymidine (DT) block and released at time 0. Nocodazole was added at 8 hours after the release. Dox was added (+Dox for Figure 2D) or not (-Dox for Figure 2C) at 14 hours after the release.

(B) Mitotic index.

(C, -Dox) and **(D, +Dox)** Extracts ere prepared at each time point and processed for western blot. For detection of p55CDC and Cmt2, ten μg of total proteins were run on SDS-PAGE. Note that the endogenous Cmt2 was not detectable under this condition. Thirty μg of total proteins for detection of securin and 50 μg for Mad2 were used.

(E) Extracts were prepared at each time point for immunoprecipitation with the antibody to Mad2 followed by western blot.

(F) FACS analysis.

(G) The cells prepared as Figure 2D were grown for 28 hours after the release from thymidine block and stained with DAPI (for DNA) and FITC-conjugated phalloidine (for cell shape). Induction of Cmt2 in the cells arrested by nocodazole resulted in a forced exit from mitosis. The majority of them exhibited larger nuclei (left panel) or multiple nuclei (middle panel). A fraction of them showed a "cut"-like phenotype (right panels). If Cmt2 was not induced, the majority of the cells exhibited condensed chromosomes with round-up cell shape, a typical phenotype of prometaphase arrest (not shown). Interphase nuclei of normal Hela cells at late G2 are shown for comparison (middle panel, inset).